

ORNITHINE TRANS-CARBAMYLASE IN POLYOMA INFECTED
MOUSE EMBRYO TISSUE CULTURE

by

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INTRODUCTION

Polyoma virus is a DNA-containing virus that produces various types of tumors in several rodents. The virus may be readily propagated in mouse embryo tissue cultures. The interaction of polyoma virus with mouse embryo tissue cultures may elicit either viral progeny after approximately twenty hours infection or transformation of cells with resulting changes in morphology and antigens. Dulbecco (1963) proposed polyoma virus infection of tissue cultured cells as an excellent model of the virus-tumor relationship. The present studies deal with infections that resulted in viral progeny.

The purpose of this study was to determine the effect of polyoma virus infection upon the ornithine transcarbamylase activity of crude extracts of mouse embryo tissue cultures and to investigate the role of ornithine transcarbamylase during the synthesis of polyoma virus particles in the above-mentioned cells. Enzyme assays in conjunction with a viral growth curve were performed to determine the effect of virus infection on the enzyme activity. Labeled citrulline (ureido- ^{14}C) pulse and pulse-chase experiments were performed to determine the involvement of the enzyme product in synthesis of viral particles. Purification of citrulline-ureido- ^{14}C labeled virus and a control experiment were performed to determine whether the label was incorporated into protein or nucleic acid polymers of the virus particles.

REVIEW OF LITERATURE

Polyoma virus was discovered independently by Gross (1953) and Stewart (1953). The virus infects small rodents such as mice, rats, hamsters, rabbits, and guinea pigs (Rowson, 1963; Stewart, 1960). The discovery was made during attempts to transmit mouse leukemia with filtrates of leukemic tissues. It was later isolated by Stewart et al., (1957), using mouse embryo tissue culture as a laboratory host. In the natural habitat of mice, the virus was thought to infect mainly through ingestion of previously contaminated grains or infectious urine (Rowe et al., 1961).

The virus was named polyoma because of the variety of tumors produced upon inoculation of the virus into mice (Stewart et al., 1958). The types of tumors described by Stanton et al., (1959) were bilateral and multilobar tumors of the parotid gland, pulmonary metastasis, renal cortical lesions, renal sarcomas, thymomas, mammary adenocarcinomas, epidermoid carcinomas, bone tumors, mesotheliomas, subcutaneous sarcomas and hemangioendotheliomas, adrenal carcinomas, and other miscellaneous types. The many types of tumors produced by one virus eliminated the concept that there had to be a multitude of viruses responsible for the great variety of tumors (Melnick, 1965). However, the ability of the virus to cause tumors does not seem essential for the survival of the virus in nature, since the appearance of tumors in nature is extremely rare, while the presence of virus is fairly common (Rowe et al., 1961).

Polyoma virus was grouped with the papovavirus group (papilloma, polyoma, and vacuolating virus) by Melnick (1962). Similarity of size and structure, the presence of double-stranded deoxyribonucleic acid,

intranuclear multiplication, ability to cause latent or chronic infections, and tumorigenic potential were the criteria originally employed to delineate this group.

Polyoma virus was the first papovavirus to be replicated in tissue culture. The ability of the virus to be propagated (Stewart et al., 1960) and assayed for plaque forming units (Dulbecco and Freeman, 1959); Winocour and Sachs, 1959) on mouse embryo tissue culture made this virus an excellent model tumor virus system as proposed by Dulbecco (1963). For this reason, polyoma virus as well as other papovaviruses have come under extensive study (Melnick, 1965).

Characteristics of Polyoma Virus

Preparations of purified polyoma virus contain two classes of particles based on their buoyant density in cesium chloride density gradients (Abel and Crawford, 1963; Crawford et al., 1962; Winocour, 1963) and sedimentation coefficients (Crawford and Crawford, 1963). One class of particles has a density of 1.29 grams per milliliter and the other has a density of 1.32 to 1.33 grams per milliliter with 140 S and 238 S sedimentation coefficients respectively. Plaque forming and transforming activities were associated with the heavier particles which contained nucleic acid cores and absorbed ultraviolet radiation in a spectrum typical of nucleoprotein. Hemagglutinating activity was associated with both the light and heavy particles. Electron micrographs showed that the lighter particles were "empty" while the heavier ones were "full" of nucleic acid (Abel and Crawford, 1963; Winocour, 1963).

The virus was found to be spherical, about 44 mu in diameter (Kahler et al., 1959; Wildy et al., 1960) with an icosahedral shell of forty-two

long, hollow capsomeres regularly disposed in a 5:3:2 symmetry on the virus surface (Almeida et al., 1966; Breese, Jr., 1964). Klug and Finch (1965) reported that the particles probably contained seventy-two capsomeres. Utilizing complete and incomplete virus, Thorne et al., (1965) concluded that the capsid alone determined the electrophoretic properties of the virus particle. The effects of physical and chemical agents on the virus were studied by Eddy et al., (1958a) and by Brodsky et al., (1959) who found the virus to be highly resistant to the environmental influences.

The DNA (Smith et al., 1960) of complete polyoma virus particles was reported to have a molecular weight of 7.5×10^6 (Crawford et al., 1962) or 3.5×10^6 (Crawford, 1964) and was isolated in a double-stranded form (Crawford, 1963). Sedimenting components of 15.5 S and 21 S were observed (Crawford and Crawford, 1963). The 15.5 S linear component resulted from the opening of the 21 S circular ring (Crawford, 1964). A DNA content of 13.4% (Winocour, 1963) and a base composition of mol % (Crawford, 1963) or 49 mol % (Weil and Vinograd, 1963) guanine plus cytosine was reported. Circular and open forms of the DNA were biologically active and possessed cytotoxic activity for mouse embryo cells and transforming activity for hamster cells (Weil and Vinograd, 1963; Crawford et al., 1964). The DNA isolated from polyoma virus was infectious for mouse embryo cells in vitro (DiMayorca et al., 1959; Smith et al., 1960) and capable of inducing tumors in vivo. Deoxyribonuclease digestion of viral DNA under controlled conditions was found to break the closed or circular polyoma DNA into "open" forms (Dulbecco and Vogt, 1963). The DNA was double-stranded (Mayor, 1961), melted at $85-95^{\circ}\text{C}$

(Crawford, 1963), and underwent semiconservative replication (Weil et al., 1966).

The virus hemagglutinated erythrocytes from hamsters, guinea pigs, humans, (type O), mice, rhesus and cynomolgous monkeys, and chickens at 4°C with nonenzymatic elution at 37°C (Eddy et al., 1958b; Sachs et al., 1959). It attached to red cells by adsorbing to the same mucoprotein receptor sites as do the influenza viruses. The red cell receptor sites for polyoma virus may be destroyed by receptor-destroying enzyme (RDE) isolated from Vibrio comma or influenza virus neurominidase (Hartley and Rowe, 1959).

Concentration and Purification of Polyoma Virus

A variety of methods have been utilized to concentrate and purify polyoma virus. Kahler et al. (1959) absorbed the virus from a crude preparation using guinea pig red blood cells, concentrated the eluted virus by centrifugation, and purified the virus in a deuterium oxide gradient. After adsorbing the virus to Dowex, Sheinin (1962) concentrated the preparation with centrifugation. Sheinin also precipitated the virus with protamine to concentrate and purify the virus. Using protamine precipitation followed by chromatography on Sephadex G75 to partition the protamine, Inglot et al. (1964) concentrated the virus by centrifugation.

After concentrating the virus by centrifugation (Winocour, 1963) purified the virus with equilibrium centrifugation in a cesium chloride density gradient. Smith et al. (1960) concentrated the virus by centrifugation, treated the preparation with enzymes (deoxyribonuclease, ribonuclease, and trypsin) to remove contaminating host materials, and purified

the final preparation in a cesium chloride density gradient. Enzyme treatment, fluorocarbon extraction, and a rubidium chloride density gradient, followed by sucrose gradient electrophoresis were used by Thorne et al. (1965) to obtain purified virus. Kazumobu (1964) purified the virus by fluorocarbon extraction followed by DEAE cellulose chromatography. Consigli et al. (1966) used ammonium sulfate precipitation for concentration, enzyme treatment to free contaminating host materials from the virus particles, and a cesium chloride density gradient to purify the virus.

The Eclipse Period

Polyoma virus has a relatively long growth cycle (18-21 hr) in tissue culture systems. Adsorption of the virus to tissue cultures occurs rapidly (Winocour and Sachs, 1960). Using labeled virus, Bourgaux (1964) observed 31% of the virus adsorbed after 30 minutes. The antigenic characteristics of the virus are related to the efficiency of adsorption (Medina and Sachs, 1963). Sheinin and Quinn (1965) reported 100% infection of tissue culture with a mutant of polyoma T virus. In contrast, Khare (1966) observed viral antigen attached to only 32% of the tissue culture after 3.0 hr adsorption with a mixture of large and small plaque mutants. The mucoprotein receptor sites destroyed by receptor destroying enzyme (RDE) are involved in the entry of the virus into the cells (Rowe, 1961). Electron microscopy of ultrathin sections of autoradiographs demonstrated polyoma virus phagocytized in cytoplasmic vacuoles (de Harven et al., 1965). Polyoma virus was observed in cell invaginations 2.0 hr after infection (Mattern et al., 1966).

Using autoradiographs, Khare and Consigli (1965) observed virus in the cytoplasm 3.0 hr after infection.

Inhibition of cell division was reported by Sheinin and Quinn (1965) as an early function of virus infection. The inhibition was detectable as early as 4.0 hr after infection. The virus is uncoated in the cytoplasm after 3.0 to 6.0 hr infection and located at the nuclear membrane after 9.0 hr infection (Khare and Consigli, 1965). Mattern et al. (1966) noted viral particles between the two nuclear membranes 8.0 hr after infection. A decrease of radiosensitivity was observed after 7.0 hr infection by Logan and Whitmore (1965).

Gershon and Sachs (1964), using puromycin inhibition, concluded that after 8.0 to 9.0 hr infection "early" protein synthesis was necessary for DNA synthesis. Benjamin (1966) reported small amounts of "early" virus specific RNA. An increase of thymidine kinase activity after 8.0 to 10.0 hr infection was demonstrated, and Sheinin (1966a) suggested that the increased activity was due to de novo synthesis of the enzyme. Dulbecco et al. (1965) noted an increase of thymidine kinase, deoxycytidine monophosphate deaminase, and DNA polymerase activities beginning 12 hr after infection. The increase of activity was not induced by capsid material. The increases of deoxycytidine monophosphate deaminase and thymidine kinase activity were due to increased rates of enzyme synthesis (Hartwell et al., 1965). Weil et al. (1966) reported virus specific RNA 12 hr after infection.

Assaying for infectious DNA, Gershon and Sachs (1964) noted polyoma virus DNA replication 12.0 to 14.0 hr after infection. Sequential FUDR inhibition indicated that DNA synthesis occurred 17.5 hr after infection

(Sheinin, 1964), but later Sheinin (1966b) observed viral DNA synthesis after only 12 hr infection by a sensitive method which utilized a methylated albumin-Kieselguhr column for separation of viral and host DNA.

Sheinin and Quinn (1965) observed an increase of viral antigens 12 hr after infection. A temperature sensitive step in the synthesis of a small plaque mutant was observed 12 to 14 hr after infection (Gershon and Sachs, 1965). The step probably involved synthesis of an enzyme or "late" protein. Weil et al. (1965) reported a psychrosensitive step which initiated the replication of polyoma virus. A full complement of proteins 14 to 16 hr after infection is necessary for viral maturation (Gershon and Sachs, 1964). A sharp increase in virus specific RNA was noted during the latter phase of virus development (Benjamin, 1966). Weil et al. (1966) noted fluorescent antibody staining viral antigens 15 to 16 hr after infection. Incorporation of H^3 -valine into immunological precipitating proteins was observed by Abo Ahmed (1966) 17 to 18 hr after infection. DNAase insusceptible particles (complete virus) appeared 18 to 21 hr after infection (Khare, 1966).

Bourgaux (1964) observed that P^{32} labeled polyoma virus became nuclear associated. Using electron micrographs (Bernhard et al., 1959; Howatson and Almeida, 1960) and autoradiography (de Harven et al., 1965; Khare and Consigli, 1965), viral synthesis was demonstrated in the nucleus. Twenty hours after infection small densely staining bundles of filaments appear in the nucleus (Mattern et al., 1966). The bundles are apparently the precursors of polyoma virus (Bernhard et al., 1959). Minowada and Moore (1963) noted de novo synthesis of viral DNA in the nuclei.

Consigli and Minocha (submitted for publication) observed that DNA

synthesis was necessary for the synthesis of "late" proteins. An increase of lipase activity and polyoma antigen synthesis was noted by Khare (1966) 19 hr after infection. Henle et al. (1959), using fluorescent antibody staining, detected viral antigens in the nuclei 48 hr after infection. Winocour and Sachs (1960), however, found nuclear fluorescent antibody staining in the nucleus 24 hr after infection. Minowada and Moore (1963) and Gershon and Sachs (1964) detected viral antigens 24 hr after infection and viral progeny 22 to 24 hr after infection. Progeny viruses were demonstrated 18 to 20 hr after infection by Sheinin (1964). Increases in total virus after 18 hr (Mattern et al., 1966), 19 to 20 hr (Minocha, 1967), and 22 hr (Winocour and Sachs, 1960) infection followed by an increase of cell-free virus 24 hr (Winocour and Sachs, 1960) after infection were reported.

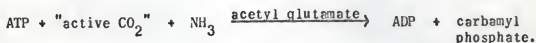
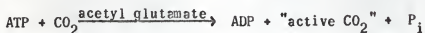
Vogt and Dulbecco (1960) did not detect cytopathic effects during the first 26 hr infection although Berecsy et al. (1961) observed cytopathic effects 2.0 to 3.0 days after infection. Sheinin and Quinn (1965) and Consigli et al. (1966) demonstrated that the "cell pool", supplied by the medium, was the source of precursors of nucleic acid synthesis and precursors of protein synthesis.

In summary, the events occurring during the eclipse period of polyoma virus are similar to other DNA containing animal viruses, such as vaccinia (Salzman et al., 1963), adenovirus (Wilcox and Ginsberg, 1963), and herpes simplex (Roizman et al., 1963).

Ornithine Transcarbamylase (Carbamoylphosphate:L-ornithine carbamoyl-transferase, EC No. 2.1.3.3)

The present concept of arginine synthesis, i.e. urea synthesis, in mammalian tissue had its beginning in the observations of Krebs and Henseleit (1932). They observed an enhancement of urea synthesis in liver slices by addition of ornithine. Krebs proposed that ornithine reacted the NH_3 and CO_2 to form citrulline. NH_3 was then condensed with citrulline to form arginine which subsequently cleaved to urea and ornithine by arginase.

Formation of citrulline from ornithine was first observed by Borsook and Dubnoff (1947) in guinea pig liver homogenates. Ornithine was condensed with carbamyl phosphate (Caravaca and Grisolia, 1960; Hall and Cohen, 1957; Metzenberg et al., 1957) by the enzyme ornithine transcarbamylase (OTCase) (Reichard, 1957; Burnett and Cohen, 1957; Joseph et al., 1963; Sato et al., 1960) in mammalian systems synthesizing citrulline. The carbamyl phosphate was probably synthesized by a two-step reaction proposed by Metzenberg et al. (1957; 1958).



Jones and Spector (1960) suggested that "active CO_2 " was a compound containing CO_2 and N-acetyl-L-glutamate. Pierard et al. (1965) designated the carbamyl phosphate synthesizing enzymes as glutamine carbamyl phosphate synthetases. Ornithine was derived from glutamate through a glutamate γ -semialdehyde intermediate (Stetten, 1951) or

from proline through Δ^1 -Pyrroline -5-carboxylic acid (Johnson and Strecker, 1962) and glutamate - γ - semialdehyde (Stetten, 1951) intermediates.

The reaction catalyzed by ornithine transcarbamylase is the first step in the synthesis of arginine and is subject to feedback inhibition and end-product repression by arginine (Gorini and Maas, 1957; Gorini, 1960; Rodgers, 1961; Rodgers and Novelli, 1962; Novick and Maas, 1961). Studies by Ratner and colleagues (1954; 1962) demonstrated that arginine formation from citrulline specifically required aspartate as well as ATP and the reaction occurred in two steps involving the intermediate formation of argininosuccinic acid (Ratner and Petrack, 1953; Ratner *et al.*, 1953a; 1953b; Ratner, 1962). The pathway of arginine synthesis is illustrated in Fig. 1 (p. 12).

MATERIALS AND METHODS

Mouse Embryo Tissue Culture Preparation

The mouse colony was obtained from Charles River Breeder, Brockton, Massachusetts. Mouse embryos (18 to 20 days) with extremities removed were trypsinized (0.25% trypsin and 0.01 M sodium phosphate buffer, pH 7.2, in 0.15 M sodium chloride). The activity of the trypsin was stopped by resuspending the cells in IX Eagle's (MEM) medium containing 10% fetal calf serum. The resulting cell suspension was seeded at a concentration of 1.2×10^6 cells per ml in IX Eagle's (MEM) medium containing 10% fetal calf serum (5.0 ml total in 60 mm petri dishes and 15 ml total in 100 mm petri dishes). Upon incubation at 37°C in a humidified CO₂

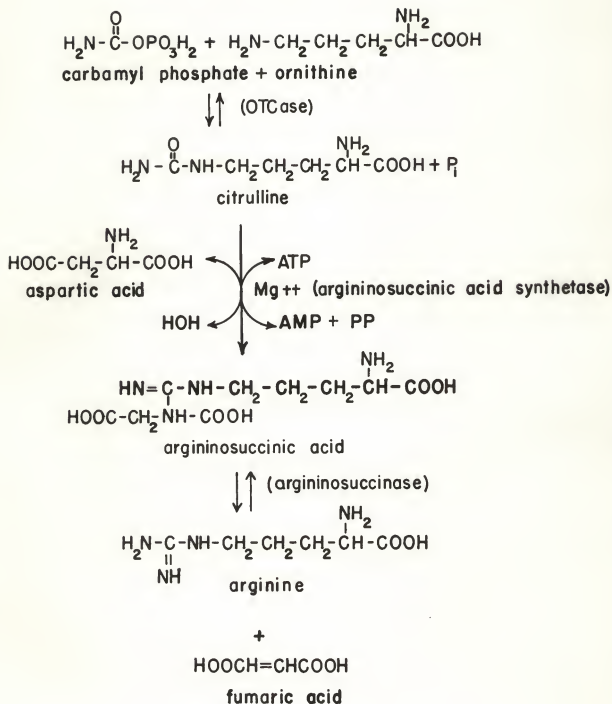


Figure 1

incubator, confluent monolayers formed after 60 to 72 hours. Media on the monolayers was removed and replaced with media containing 5% fetal calf serum after 72 and 96 hours in an attempt to obtain monolayers in a state of contact inhibition. All experimental monolayers were of this type unless stated otherwise.

Determination of Plaque-Forming Units (PFU)

Plaque-forming units of polyoma virus samples were determined by a modification of Dulbecco's agar overlay method (Dulbecco, 1952; Dulbecco *et al.*, 1954; Winocour and Sachs, 1960; Dulbecco and Freeman, 1959). Immediately prior to the formation of confluent monolayers of primary mouse embryo tissue in 60 mm Falcon Plastic Tissue Culture Dishes, 0.5 ml of polyoma virus samples of Log_{10} dilutions in IX Eagle's (MEM) medium were absorbed for 2.5 hr at 37°C in a humidified CO_2 incubator. Eight milliliters of Adeno medium (Minocha, 1967) containing 5% fetal calf serum and 1% purified Difco agar were added to the dishes. Seven days after absorption the dishes were fed with 3.0 ml of the preceding medium to maintain the monolayers. Two milliliters of the preceding medium containing 0.01% neutral red were added 12 days after absorption and the plaques were enumerated after 24 and 48 hours.

Virus Propagation

Strain (PyD) of polyoma virus was obtained from Dr. Vittorio Defendi, Wistar Institute, Philadelphia, Pennsylvania. The strain exhibited approximately 80% large and 20% small plaques.

Virus stocks of 20,000 hemagglutination (HA) units per ml and

3.2×10^9 PFU per ml were obtained by infecting confluent mouse embryo monolayers (Eddy et al., 1956) in 32 oz. glass culture bottles with 1.0 ml of virus having an HA titer of 5000. After a 3 hr virus absorption, IX Eagle's (MEM) medium containing 5% fetal calf serum was added and the monolayers were incubated 5 to 7 days (37°C). At the time of harvest the phenol red indicator appeared orange-red. The cells exhibited extensive cytopathic effects, but few cells had released from the glass.

The monolayers were polished into the media and the suspension was centrifuged at $250 \times g$ for 0.5 hr at 5°C . The supernatant was discarded after an HA assay yielded less than 2 HA units per ml. The sediment was resuspended in a "lysing buffer solution" (0.1 M NaCl, 0.1 M Tris-HCl buffer, pH 8.0) and lysed with a Servall Omnimixer treatment (40 ml cup) for five minutes (0°C). Twenty units per milliliter of RDE (Receptor Destroying Enzyme, Microbiological Associates, Bethesda, Maryland) and IX antibiotic concentration (Minocha, 1967) were incubated with the lysate at 37°C for 24 hr followed by 2.0 hr of incubation with 0.1 mg per ml trypsin (2X crystalline, salt free, General Biochemicals, Chargin Fall, Ohio). The trypsin was inactivated with an equivalent weight of soya bean trypsin inhibitor (5X crystalline, Nutritional Biochemicals Corporation, Cleveland, Ohio) and the suspension was centrifuged at $1300 \times g$ for 30 min (5°C) to remove the cellular debris. The supernatant was adjusted with a NaCl solution to yield a final concentration of 0.15 M NaCl, and the stock virus was stored (-20°C).

Determination of Hemagglutination Titer (HA)

Hemagglutinating activities of polyoma virus samples were determined by the ability of polyoma virus to hemagglutinate guinea pig red blood cells (Eddy et al., 1958b; Sachs et al., 1959). Serial two-fold dilutions of virus samples in plastic dilution trays (Linbro Chemicals) were mixed with an equal volume of 0.75% washed guinea pig red blood cells in phosphate buffered saline (0.01 M sodium phosphate buffer in 0.15 M sodium chloride) and incubated 3.0 hr (5⁰C). The final dilution which exhibited positive hemagglutination was assumed to contain one HA unit or approximately 500,000 virus particles (Eddy et al., 1958b; Medina and Sachs, 1961).

Preparation of Media

Eagle's (MEM) medium was prepared by the method specified by Minocha (1967).

Preparation of Enzyme Extract

Crude extracts of monolayers were used for the enzyme assay. Mouse embryo cell cultures were policed into phosphate buffered saline and centrifuged at 1000 x g for 10 minutes. The supernatant was decanted, and the pellet was resuspended in an appropriate aliquot of neutral saline. A sample was removed for cell enumeration (Whiteline hemocytometer) and the suspension of cells was subjected to disruption in a Servall Omnimixer. The suspension of treated cells was centrifuged at 250 x g for 10 min to remove the cells which were not disrupted and the supernatant was designated as crude enzyme extract. Manipulations from the

harvest to the crude enzyme extract were performed at 5°C.

Enzyme Assay

Ornithine transcarbamylase (OTCase) was assayed by a modification of the method of Archibald, (1945), a colorimetric assay for citrulline (Koritz and Cohen, 1954). The enzyme reaction mixture contained:

- a) 2.0 ml of enzyme (crude extract)
- b) 0.5 ml of ornithine (50 uM/ml.)
- c) 0.5 ml of carbamyl phosphate (30 uM/ml) in 0.1 M Tris Acetate Buffer pH 8.0
- d) 0.1 ml of $MgCl_2$ (1.0 uM/ml.)

All components except CAP were mixed at 5°C. Carbamyl phosphate was added to start the reaction and a sample was immediately removed (0 time) for later assay. The reaction was placed in a 37°C water bath and incubated 1.0 hr. Immediately after removing samples for assay, 2.5 ml of water were added to assay tubes (Kimax Raysorb Culture tubes, Owens-Illinois, Toledo, Ohio) followed by 2.0 ml of acid mixture (concentrated sulfuric-phosphoric acids 1:3, v/v) to quench reactions. Quenched samples were stored in an ice bath until all samples could be assayed. To complete assays 0.75 ml of 1% aqueous diacetylmonoxime was added. Components of assays were mixed immediately after addition of acid mixture and also after addition of diacetylmonoxime. The mixtures were placed in boiling water for 15 min., cooled, and the absorbance determined with a Klett-Sommerson colorimeter using a Corning #54 filter. Standard concentration curves of citrulline were determined with each assay.

Preparation of Scintillation Fluid

Fifteen grams of PPO (2,5-Diphenyloxazole, Scintillation Grade,

Packard Instrument Company, Inc., La Grange, Ill.) and 240 g of naphthlene were dissolved in 1080 ml of toluene, followed by an equal volume of dioxane. The mixture was completed with 648 ml of absolute ethanol.

Preparation of CsCl Density Gradients

Cesium chloride (Fairmount Chemical Co., Inc. Newark, N. J.) solutions of 1.20, 1.23, 1.25, 1.28, 1.30, 1.33, 1.35, 1.38, and 1.40 g per ml densities were prepared in 0.01 M Tris-HCl buffer (pH 7.2) from a 1.60 g per ml stock solution. Equal volumes of the various densities were sequentially layered in a centrifuge tube and the virus preparations were layered on the top layer (1.20 g per ml). Densities of prepared solutions and of collected fractions were determined with a Bausch and Lomb refractometer.

Lytic Infection Cycle

Polyoma virus (0.1 ml of 3.2×10^9 pfu per ml stock) was absorbed to contact inhibited mouse embryo monolayers in 60 mm dishes for 3.0 hr (Winocour and Sachs, 1960). The monolayers were washed three times with IX Hank's balanced salt solution to remove nonabsorbed virus and IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum was added for maintenance. At appropriate intervals, three dishes were pelleted into the supernatant and freeze-thawed three times. This suspension was assayed for pfu activity.

Determination of Enzyme Activity During the Eclipse Phase

Polyoma virus (0.1 ml of 3.2×10^9 pfu per ml stock) was absorbed

to contact inhibited monolayers in 60 mm dishes for 3.0 hr. IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum was added for cell maintenance. At various intervals during the lytic cycle, the monolayers were policed into phosphate buffered saline and processed to assay for enzyme activity.

Citrulline-ureido-¹⁴C Pulse

Contact inhibited monolayers in 60 mm dishes were infected with 3.2×10^8 pfu per dish. After 3.0 hr of infection, IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum was placed upon the monolayers and the infection was allowed to proceed until the time of the pulse. One hour prior to harvesting, the media was removed from three dishes and replaced with 1.0 ml of IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum and 1.5 uC per ml of Citrulline-ureido-¹⁴C (New England Nuclear Corp. Boston, Mass.). After 1.0 hr of exposure to the label, the monolayers were policed into PBS, centrifuged to separate the cells, and washed once with phosphate buffered saline to remove extracellular label. The cells were then resuspended in distilled water and precipitated with a final concentration of 5% trichloroacetic acid. The cell precipitate was washed twice with 5% trichloroacetic acid (Piez and Eagle, 1958) and the specific radioactivity was determined. Protein was assayed by the method of Lowry *et al.* (1951).

Citrulline-ureido-¹⁴C Pulse Chase

Contact inhibited mouse embryo monolayers in 100 mm dishes were infected with 9.6×10^8 pfu per dish. After 3 hr of infection IX

Eagle's (MEM) medium containing 5% dialyzed fetal calf serum was placed on the cells for maintenance during the infection. At appropriate intervals during infection the media was removed from four dishes and replaced with 2.5 ml per dish of the same media containing 2.5 μ C of citrulline-ureido- 14 C. After 2.0 hr of exposure to the label the radioactive media was removed from the monolayers. The monolayers were washed twice with IX Eagle's media and 8.0 ml of IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum and 10 μ g per ml of non-labeled citrulline. The non-labeled citrulline was used to chase the label into the polymers to be isolated. After a total of 50 hr of infection, the various intervals were policed from the dish and frozen-thawed three times to release the virus. Twenty units per milliliter RDE (20 units per ml) was added after adjusting the suspension to approximately pH 8.0 with 8% NaHCO_3 solution. After incubating 24 hr (37°C), the debris was removed from the supernatant by centrifugation at $80,000 \times g$ for 3.0 hours. The pellet was resuspended in 0.1 M Tris HCl buffer containing 0.15 M NaCl and 20 μ g per ml of DNAase (Deoxyribonuclease, bovine pancreas, B grade, Calbiochem, Los Angeles, Calif.) and RNAase (Ribonuclease, bovine pancreas A grade, Los Angeles, Calif.) were added in the presence of 3.0×10^{-3} μM MgCl_2 and incubated for 1.0 hr (37°C). The action of the nucleases was terminated by trypsin (0.1 mg per ml) treatment for thirty minutes (37°C) followed by an equivalent amount of trypsin inhibitor. The enzymes were removed by centrifugation at $156,000 \times g$ for 1.5 hour. The pellet of virus was resuspended in 0.1 M Tris-HCl containing 0.15 M NaCl and placed upon a preset CsCl density gradient (range-1.20 to 1.40 g per ml). The gradient was centrifuged at

156,000 x g for 18 hours. The fractions characterized by HA were examined for radioactivity and specific radioactivity was calculated.

Purification of Citrulline-ureido-¹⁴C Labeled Polyoma Virus

Confluent mouse embryo monolayers in 60 mm dishes were infected with 3.2×10^8 PFU per dish. After 3 hr of infection, IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum and 1.0 uC per ml of citrulline-ureido-¹⁴C was placed on the monolayers. On the third day of infection 1.0 ml of IX Eagle's (MEM) medium containing 5% dialyzed fetal calf serum was added for culture maintenance. On the sixth day of infection the monolayers were policed, pooled, centrifuged at 1000 x g for 20 min and resuspended in 30 ml of 0.1 M Tris-Acetate buffer (pH 8.0) in 0.1 M NaCl. The suspension was lysed by Omnimixer treatment in a 40 ml cup at 100% power for five minutes. The lysed cells were incubated for 24 hr (37°C) with RDE (20 units per ml) followed by incubation 1.0 hr (37°C) with trypsin (0.1 mg per ml). An equivalent amount of trypsin inhibitor was added to stop the enzyme action. The enzyme treated suspension was centrifuged at 2000 x g for 20 min to sediment large cellular debris. Enzyme treatment was repeated on the sediment and the second supernatant was combined with the first supernatant. The virus was concentrated by centrifuging the supernatant at 80,000 x g for 3.0 hr and resuspending the pellet in 0.01 M Tris-HCl buffer (pH 7.2) in 0.15 M NaCl. The suspension was incubated with DNAase and RNAase (20 ug per ml of each) for 1.0 hr (37°C). Enzyme action was stopped by incubation for 30 min with trypsin (0.1 mg per ml) followed by addition of an equivalent weight of trypsin inhibitor. The enzymes were removed by centrifuging the virus

suspension at $156,000 \times g$ for 2.0 hours. The pellet was resuspended in the preceeding Tris-HCl buffer and applied to a preset CsCl density gradient. The gradient was centrifuged at $156,000 \times g$ for 18 hr and fractionated with a Buchler Instruments gradient fractionator. Fractions characterized as polyoma virus by HA activity and density were dialyzed for 24 hr against 0.01 M Tris-HCl buffer (pH 7.2). The suspension was placed on a second density gradient and centrifuged as before. Fractions were again collected and characterized by PFU activity, HA activity, density, and radioactivity.

RESULTS

Method of Lysing Mouse Embryo Tissue Culture Cells

Primary mouse embryo tissue cultures were policed, pooled in neutral saline, and subjected to various intervals of Servall Omnimixer treatment in a 40 ml cup at a 100% power setting. Table I illustrates that up to 36 sec of Omnimixer treatment resulted in increased enzyme activity of the crude enzyme extract. Additional time of treatment resulted in decreased activity of the crude enzyme extract. All enzyme extracts of mouse embryo tissue culture cells used in the following experiment were made with 36 sec of Omnimixer treatment.

TABLE I Relationship of Enzyme Activity in Extracts After Various Intervals of Servall Omnimixer Treatment.

Time (seconds)	μ M citrulline synthesized (per 2×10^6 cells / hr)
12	0.129
24	0.152
36	0.169
48	0.137
60	0.122

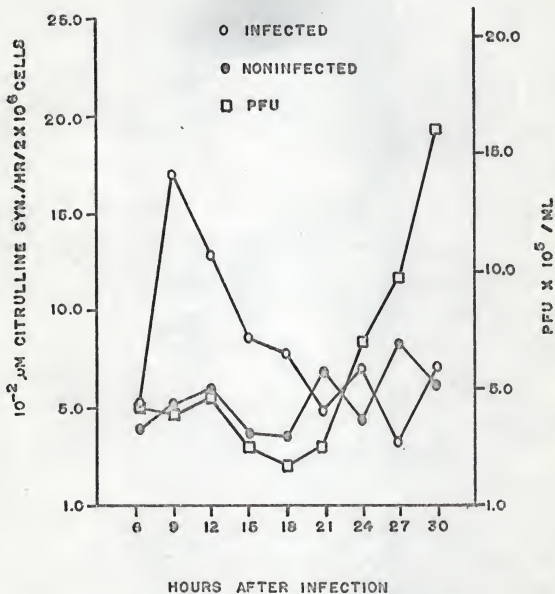
Relationship Between OTCase Activity and the Appearance of Complete Virus

To determine if an increase of OTCase activity occurred in polyoma infected mouse embryo tissue culture, an enzyme assay was performed on infected and noninfected cells at various intervals after infection. PFU assays were included at the same intervals to determine the chronological relationship of enzyme activity and appearance of complete virus. Plate I (p. 24) illustrates that PFU's increased after 21 hr infection, while enzyme activity increased to a peak of activity after 9.0 hr infection with a subsequent decrease of activity to the level of the noninfected cells by 21 hours. An approximate three-fold increase of OTCase activity occurred after 9 hr infection. This increase of OTCase activity occurred 12 to 15 hr prior to the appearance of complete virus.

EXPLANATION OF PLATE I

OTCase Activity During a Lytic Cycle of Polyoma Virus Infection

Polyoma virus was absorbed for three hours to mouse embryo tissue cultures. Samples of infected and noninfected cells were assayed for PFU and enzyme activity after various intervals of infection.

OTCase ACTIVITY DURING A GROWTH
CYCLE

Citrulline-ureido-¹⁴C Pulse

In order to determine if the observed increase in enzyme activity was contributing to polymer synthesis, a labeled citrulline pulse experiment was performed. At various time intervals after infection, monolayers were exposed to citrulline-ureido-¹⁴C. After 1.0 hr of exposure to the label, the monolayers were harvested, and the trichloroacetic acid soluble fraction (amino acid pool) was removed. The specific radioactivity of the acid insoluble fraction (polymers) was determined. (Plate II, p. 27).

An increase of label incorporation was observed at 18 hr after infection. This was 9.0 hr after increased enzyme activity and 3.0 hr prior to the appearance of complete virus.

Citrulline-ureido-¹⁴C Pulse Chase

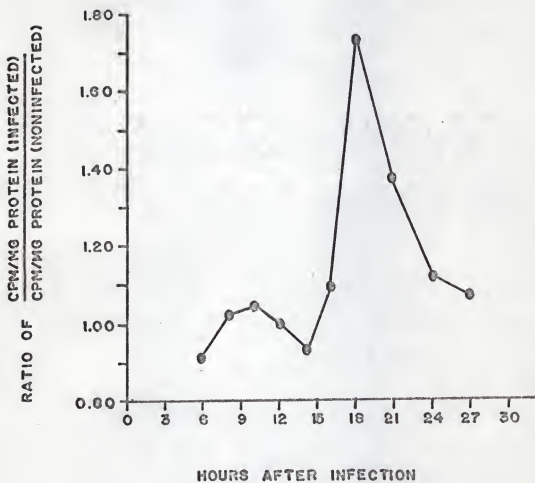
In order to establish the relationship of the increased activity observed in the pulse experiment with the virus particle, a pulse chase experiment was performed. At various intervals during a virus growth cycle, the mouse embryo tissue cultures were exposed to 2.0 hour-pulses of labeled citrulline. At the end of the pulse, the monolayer was washed once with Eagle's medium and fed with Eagle's medium containing nonlabeled citrulline. Each point was then allowed to proceed to 50 hr infection. The samples were harvested; the virus was eluted and purified; and the specific radioactivity determined.

Plate III (p. 29) illustrates the observed increase of incorporation previously noted occurred in the virus particle after 18 to 20 hr infection or about 2.0 hr before the appearance of progeny virus.

EXPLANATION OF PLATE II

Citrulline-ureido-¹⁴C Pulse

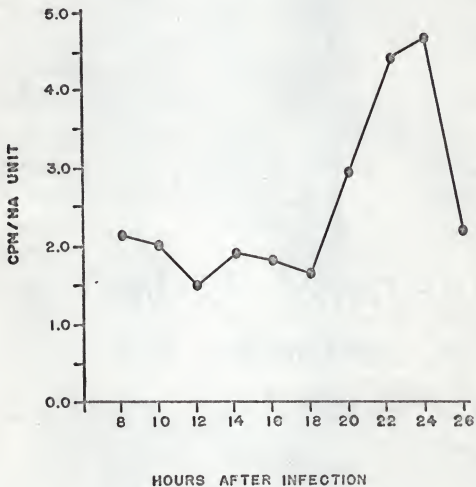
Mouse embryo monolayers were exposed to a citrulline-ureido-¹⁴C pulse at various intervals during a lytic cycle and the specific radioactivity of the incorporated label was determined.

CITRULLINE-UREIDO- ^{14}C PULSE

EXPLANATION OF PLATE III

Citrulline-ureido-¹⁴C Pulse Chase

Mouse embryo monolayers were exposed to a citrulline-ureido-¹⁴C pulse followed by a cold citrulline chase at various intervals during a lytic cycle. After a total of 50 hr infection, the progeny virus was purified and the specific radioactivity of the various virus samples were determined.

CITRULLINE-UREIDO- ^{14}C PULSE CHASE

Purification of Citrulline-ureido-¹⁴C Labeled Polyoma Virus

Because the previous experiment demonstrated that the label was incorporated into the virus particle, it was necessary to examine the characteristics of the incorporation of the label in the virus particle to eliminate the possibility of the ureido-¹⁴C group metabolizing to a new carbon skeleton. The virus was grown in the presence of labeled citrulline, eluted from the host cell, and concentrated by centrifugation. The concentrated virus was treated with RNAase, DNAase, and trypsin and subjected to a CsCl density gradient. The fractions characteristic of polyoma virus (HA, PFU, and density) were dialyzed and again subjected to a second CsCl density gradient.

Plate IV (p. 32) illustrates the data obtained from the second density gradient. The label appeared in both the complete and incomplete fractions. The complete fraction had a bouyant density of 1.34 g per ml and the greatest amount of PFU and HA activity. The incomplete band had a bouyant density of 1.30 g per ml and contained approximately five percent of the PFU activity of the complete band, while exhibiting a higher radioactivity and HA activity.

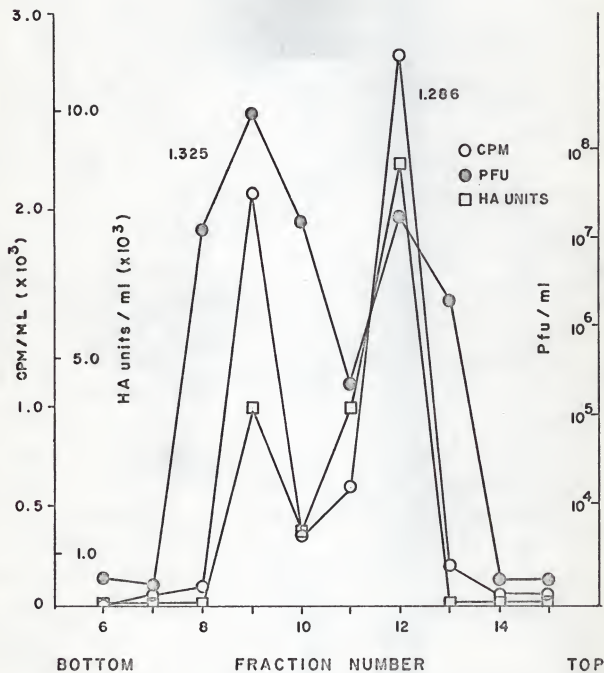
Determination of Host Contamination of Purified Viral Preparations

A possibility existed that material from the host was contaminating the virus-characterized fractions from the second density gradient. To eliminate this possibility mouse embryo tissue was cultured in the presence of an amino acid - ¹⁴C mixture to obtain labeled host proteins. A second tissue culture was infected with polyoma virus to obtain virus

EXPLANATION OF PLATE IV

Citrulline-ureido-¹⁴C Labeled Polyoma Virus Purification

Polyoma virus was grown in the presence of citrulline-ureido-¹⁴C and purified by subjecting the preparations to two consecutive density gradients after enzyme treatment. Fractions of the second gradient were assayed for radioactivity, HA, and PFU activities.

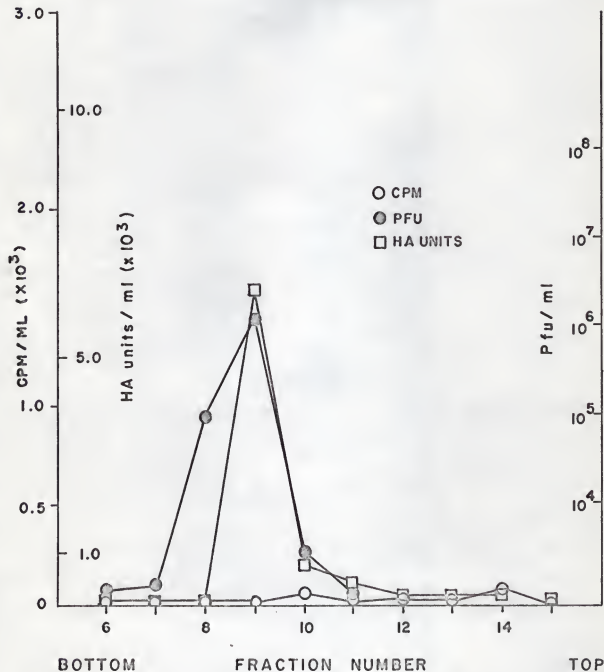
CITRULLINE-UREIDO- ^{14}C LABELED PV

EXPLANATION OF PLATE V

Host Contamination of Purified Viral Preparations

Host cells cultured in the presence of an amino acid- ^{14}C mixture were mixed with virus infected cells. The resulting mixture was purified by subjecting the preparation to two consecutive density gradients after enzyme treatment. Fractions of the second gradient were assayed for radioactivity, HA, and PFU activities.

¹⁴C-Amino Acid Mixture labeled Host Cells



which was closely associated with host material, but containing no label in the virus proteins. The tissue cultures were harvested and mixed; and the preceeding purification procedure was performed.

Plate V (p. 34) illustrates the data obtained from the second density gradient. Although there was a total of approximately 10^6 cpm in the virus-labeled host mixture, the final purified virus preparation contained essentially no radioactivity (2 cpm above background). Thus the possibility that contaminating host materials were contributing a significant amount of radioactivity to the virus characterized fractions was eliminated.

DISCUSSION

OTCase in Tissue Culture

Ornithine transcarbamylase control mechanisms have been well studied in microorganisms. OTCase was found to be subject to end-product induction and repression by arginine (Rogers and Novelli, 1962; Gorini and Maas, 1957; Gorini, 1960; Novick and Maas, 1961; Rogers, 1961; Ravel *et al.*, 1959; Bastarrachea and Ortega, 1967; Bernlohr, 1966). Citrulline synthesis was also found to be controlled indirectly by regulation of ornithine synthesis from glutamic acid in microorganisms (Vyas and Maas, 1963). However, mechanisms of enzyme control of arginine synthesis in mammalian tissues have not been studied as extensively as in microorganisms (Meister, 1965). Pardee and Wilson (1963) suggested that control mechanisms other than end-product induction

and repression such as activations, inactivations, stimulations, and inhibitions were active in mammalian enzyme systems.

However, Schimke (1962; 1964) demonstrated repression and induction of argininosuccinate synthetase and lyase in tissue cultures. In addition OTCase repression and induction by arginine was reported in intact animal studies (Civen et al., 1967). Ornithine transcarbamylase was reported in a variety of mammalian tissues (Jones et al., 1961; Burnett and Cohen, 1957; Reichard, 1957; Smith and Reichard, 1956; Caravaca and Grisolia, 1960), but investigators have noted that citrulline and not ornithine substituted for arginine in several established cell lines (Morgan et al., 1958; Tytell and Neuman, 1960; Manson and Thomas, 1960; Tedesco and Mellman, 1967). Schimke, (1964), suggested that established cell lines have no OTCase activity, and the activity observed by some investigators was due to PPLO contamination of the cell lines. The media used in the present investigation contained kanamycin sulfate (Pollock et al., 1960) to eliminate the possible role of PPLO's in the OTCase activity observed. In contrast, Shedden and Cole, (1966) reported the growth of certain PPLO strains in the presence of high concentrations of kanamycin. However, the use of primary tissue cultures would make the possibility of contamination with kanamycin resistant PPLO's even less probable.

Miedema and Kruse (1965) noted pronounced metabolic differences between pre- and post-confluent cultures. In addition, Sato et al. (1960) observed that primary cell cultures exhibited OTCase activity. The cultures lost the activity gradually with the death of nondividing cells within the primary culture. Thus it was probable that OTCase

activity could be involved in polyoma virus infection of primary tissue culture.

Relationship of OTCase Activity and the Appearance of Complete Virus

The comparison of OTCase specific activities in noninfected and infected mouse embryo monolayers at various intervals during a growth cycle indicated that in some manner polyoma virus infection elicited an increase in activity of OTCase after 9.0 hr infection. This increase was 12 hr prior to the increase of progeny virus, suggesting that the enzyme might be involved in the synthesis of "early protein" (Gershon and Sachs, 1964). Studies are in progress to elicit the mechanisms of control of OTCase activity in mouse embryo tissue cultures and the effect of polyoma virus infection on the control mechanisms of the enzyme.

Citrulline-ureido-¹⁴C Pulse

Citrulline (synthesized by OTCase) or a derivative of citrulline may have not been utilized after 9.0 hr infection. The citrulline or a derivative might have remained in a "cellular pool" (Consigli *et al.*, 1966; Darnell and Eagle, 1958; Darnell and Levintow, 1960; Eagle and Piez, 1962) until the need for citrulline as a precursor for structural polymers of the virus arose.

An increase of incorporation of citrulline-ureido-¹⁴C into a trichloroacetic acid precipitable fraction (Darnell, 1965; Piez and Eagle, 1958) after 18 hr infection immediately prior to the appearance of complete virus, suggested involvement of citrulline or a derivative in the synthesis of structural polymers (capsids) of the virus.

Abo-Ahmed (1966) also noticed increased incorporation of labeled valine into trichloroacetic acid precipitable and immunological precipitable proteins after 18 hr infection. These data would suggest that citrulline was not involved to a great extent in the synthesis of "early proteins" (Gershon and Sachs, 1964).

However, trichloroacetic acid would precipitate host and viral polymers simultaneously; therefore, the observed increase of incorporation could have been due to stimulations of synthesis of host polymers. Thus the pulse experiment did not demonstrate the actual involvement of OTCase product (citrulline) in the synthesis of the viral particle.

Citrulline-ureido- ^{14}C Pulse Chase

The incorporation of citrulline-ureido- ^{14}C into virus precursors followed by integration of the precursors into a viral particle during the chase period demonstrated that the product of OTCase was involved in the synthesis of a precursor of a structural polymer of polyoma virus. This incorporation began at approximately 18 hr after infection and occurred just prior to the appearance of complete virus. Thus citrulline or a derivative probably remained in a "cellular pool" from 9.0 to 18.0 hr after infection.

A possibility existed that the labeled ureido group of citrulline was transformed to an alternate carbon skeleton, such as ureidosuccinic acid. The reversal of the OTCase reaction could produce ornithine and radioactive carbamyl phosphate (Meister, 1965) which in turn could be utilized by aspartic transcarbamylase to produce ureidosuccinate (Reichard, 1957; Smith and Reichard, 1956). Ureidosuccinate could then

be utilized to synthesize pyrimidines for incorporation into nucleic acids.

Purification of Citrulline-ureido-¹⁴C Labeled Polyoma Virus

The labeling of both fractions (1.30 and 1.33 g / ml) of cesium chloride density gradients characterized by HA, demonstrated that the label was present in the protein fraction of the virus and very little of the label was present in the nucleic acid fraction. If the label had been in the nucleic acid portion of the virus, the incomplete fraction (1.30 g / ml) of the density gradient would have exhibited only 5 to 10% of the radioactivity of the complete fraction (1.33 g / ml). Since the nucleic acid synthesis preceded the viral capsid synthesis (Consigli et al., submitted for publication), the nucleic acid synthesis would tend to deplete the "cellular pool" of radioactive citrulline prior to the uptake of amino acids for protein synthesis and the radioactive content of the fractions would be proportional to the plaque-forming ability of the specified fraction. Results of this nature were obtained using labeled ureidosuccinate in an identical experiment (unreported data). Minocha (1967) using H³ thymidine labeled purified virus preparations also observed much less radioactivity in the incomplete fraction as compared to the complete fraction. The ureidosuccinate and thymidine are both precursors of nucleic acid synthesis.

However, more radioactivity from labeled citrulline appeared in the incomplete fraction as compared to the complete virus fraction. Hemagglutinating ability of the incomplete fraction exhibited the same characteristic ratio, further demonstrating that the radioactivity was predominately

associated with the protein fraction and not the nucleic acid fraction of the virus.

Incorporation of Citrulline Into the Virus Particle

The preceeding experiments did not elicit the form of the citrulline-ureido- ^{14}C label in the incorporated state. Three possibilities of radioactive label incorporation and final state of the incorporated label exist: 1) The label could be incorporated as citrulline (Rogers, 1958; Rogers and Simmonds, 1958; Smith and Young, 1955). 2) The label could be metabolized to arginine. The labeled ureido carbon would remain in the arginine molecule if arginine was synthesized by established pathways (Ratner, 1962) and the label could then be incorporated into a viral polymer. 3) Radioactive arginine (synthesized from labeled citrulline by the host cell) could be incorporated into a viral polymer and then converted back to citrulline by a specific enzyme (Szökörenyi *et al.*, 1954). Possibilities 2) and 3) contain arginine as a precursor to incorporation. These alternatives seem improbable since the Eagle's (MEM) medium used in the preceeding experiments contained an excess of arginine (Eagle, 1959). The arginine in the medium would probably dilute the "cellular pool" of radioactive arginine produced from the labeled citrulline. Experiments are in progress to elicit the form of the citrulline-ureido- ^{14}C label during incorporation and the final form of the incorporated label in polyoma virus particles.

SUMMARY

The involvement of OTCase activity in polyoma virus infection of mouse embryo tissue culture was studied. The OTCase activity was observed to increase 9.0 hr after infection. Pulse and pulse-chase experiments utilizing citrulline-ureido- ^{14}C demonstrated the incorporation of the OTCase product, citrulline, into the virus particle. A purification of labeled virus particles indicated that the label was in the protein fraction of polyoma virus; thus it was concluded that OTCase is involved in the synthesis of polyoma virus particles.

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ORNITHINE TRANS-CARBAMYLASE IN POLYOMA INFECTED
MOUSE EMBRYO TISSUE CULTURE

by

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The role of an enzyme, ornithine transcarbamylase, (OTCase), in protein precursor synthesis was investigated in polyoma infected mouse embryo tissue culture.

Assays for ornithine transcarbamylase were performed at various intervals during a growth cycle to determine the chronological relationship of enzyme activity and synthesis of complete virus particles. Infected tissue cultures exhibited a three-fold increase of enzyme activity nine hours after infection with a rapid decrease of activity to the noninfected level of activity.

Infected and noninfected tissue cultures were exposed to one hour pulses of citrulline-ureido- ^{14}C , and the rate of incorporation was examined during a growth cycle to determine the relationship of increased enzyme activity and the utilization of the enzyme product. Approximately a two-fold increase of incorporation was observed after eighteen hours infection. This increase of incorporation occurred just prior to the appearance of progeny virus particles.

A two hour citrulline-ureido- ^{14}C pulse, followed by a nonlabeled citrulline chase period, which allowed complete virus particles to be synthesized, was performed. The virus particles were purified and examined for specific radioactivity. After twenty to twenty two hours infection, an increase of specific radioactivity was observed in purified virus particles indicating that the label from citrulline contributed to the synthesis of polyoma virus particles.

A CsCl density gradient of C^{14} citrulline labeled polyoma virus was examined. The label appeared in both the complete (1.33 g / ml) and incomplete (1.30 g / ml) fraction indicating that the label from citrulline was in the protein fraction of the virus.